BOR Papers in Press. Published on December 6, 2006 as DOI:10.1095/biolreprod.106.056040

The Influence of Nuclear Content on Developmental Competence of Gaur X Cattle Hybrid In Vitro Fertilized and Somatic Cell Nuclear Transfer Embryos[1]

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Short Title: Nuclear-cytoplasmic interaction in hybrid embryos

Summary Sentence: Xenomitochondrial homoplasmy contributes to the poor developmental outcome observed in interspecies SCNT embryos between closely related *Bos* species.

[1]Supported by grants from the Natural Sciences and Engineering Research Council of Canada, Ontario Graduate Scholarships and the Canada Research Chair Program.

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ABSTRACT

In non-domestic and endangered species, the use of domestic animal oocytes as recipients for exotic donor nuclei causes the normal pattern of cytoplasmic inheritance to be disrupted, resulting in the production of nuclear-cytoplasmic hybrids. Evidence suggests that conflict between nuclear and cytoplasmic control elements leads to a disruption of normal cellular processes, including metabolic function and cell division. This study investigated the effects of nuclear-cytoplasmic interactions on the developmental potential of interspecies embryos produced by in vitro fertilization and somatic cell nuclear transfer: cattle x cattle, gaur x cattle, hybrid x cattle. Cattle control and hybrid embryos were examined for development to the blastocyst stage and blastocyst quality, as determined by cell number and allocation, apoptosis incidence, and expression patterns of mitochondria-related genes. These analyses demonstrated that a 100% gaur nucleus within a domestic cattle cytoplasmic environment was not properly capable of directing embryo development in the later preimplantation stages. Poor blastocyst development accompanied by developmental delay, decreased cell numbers, and aberrant apoptotic and related gene expression profiles, all signs of disrupted cellular processes associated with mitochondrial function, were observed. Developmental potential was improved when at least a portion of the nuclear genome corresponded to the inherited cytoplasm indicating that recognition of cytoplasmic components by the nucleus is crucial for proper cellular function and embryo development. A better understanding of the influence of the cytoplasmic environment on embryonic processes is necessary before interspecies somatic cell nuclear transfer can be considered a viable alternative for endangered species conservation.

INTRODUCTION

The success of somatic cell nuclear transfer (SCNT) in domestic and laboratory animals demonstrates the tremendous potential for its use in the preservation and dissemination of genetic material. The ability to conserve an individual's entire genome for contribution to future offspring presents an appealing alternative for the preservation of genetic diversity in non-domestic and endangered species. In many of these species, the requirements for domestic animal oocytes as recipients for exotic donor nuclei has resulted in the production of a new type of hybrid. Interspecific / intergeneric nuclear-cytoplasmic hybrids have been reported to produce preimplantation embryos and, in some cases, establish pregnancies [1-4]. The domestic cattle (*Bos taurus*) cytoplasm has been shown to support the nuclei of various species to the early cleavage stages and beyond, suggesting that it has the ability to reprogram, at least partially, diverse donor nuclei past maternal-embryonic transition [2, 4-6].

In recent years there has been an increasing interest in the role of nuclear- cytoplasmic compatibility in embryo development in vitro. This is of particular importance in SCNT embryos where the nucleus and cytoplasm are derived from unrelated individuals or species. Studies show that cytoplasmic components inherited from the donor cell or recipient ooplasm influence developmental outcome. Centrosomes, essential for establishing the mitotic spindle, cell polarity and cell division, are introduced by the donor cell into the reconstructed embryo [7]. Centrosome defects create errors during cell division that result in chromosome instability and aneuploidies [8]. Abnormal centrosome replication, distribution and function have been associated with poor developmental potential of SCNT embryos [9]. Mitochondria, on the other hand, are inherited primarily from the recipient ooplasm with a small number provided by the donor cell. The role of

mitochondria in energy production makes them a critical factor in normal gamete and embryo development [10-11]. Alterations in structure, location and mtDNA copy numbers are correlated with changes in ATP levels, which can impede the embryo's ability to undergo the various energy-demanding events necessary to achieve blastulation [12-13]. In SCNT embryos, nuclear-mitochondrial interaction is complicated by the fact that the donor nucleus has to function properly in either a heteroplasmic environment consisting of two mitochondrial lineages or a homoplasmic environment consisting of a mitochondrial lineage unrelated to the nuclear genome. Heteroplasmy has been associated with poor development outcome in SCNT embryos and possibly with the phenotypic variation observed in cloned offspring [14]. Examination of heteroplasmic SCNT embryos produced from *Bos taurus* oocytes and donor cells of different mtDNA haplotypes indicated that the type of recipient cytoplasm affected the developmental ability of the embryo and the fetus, with changes in fetal phenotype and fetal cell metabolism being observed [15]. Xenomitochondrial homoplasmy, when the mitochondria of one species interact with the nucleus of an unrelated species results in serious metabolic disruption due to a reduction in mitochondrial respiratory function [16-18].

It is thought that species that hybridize naturally are more likely to have success with interspecies SCNT since the production of living hybrid offspring would suggest that some nuclear- cytoplasmic compatibility exists between the two species. The gaur (*Bos gaurus*) is closely related to domestic cattle, having diverged fairly recently in evolutionary time [19]. As a result, the gaur has been bred successfully with *Bos taurus* [20], *Bos indicus* [21], and *Bos indicus-Bos taurus* crosses [22]. An attempt at interspecies SCNT using gaur donor nuclei and domestic cattle oocytes produced several pregnancies and one live offspring, which subsequently died due to unrelated causes [2]. However, the low blastocyst rate (12.5%) observed in that study

suggests that the communication, or lack thereof, between the gaur nucleus and domestic cattle cytoplasm may be influencing the developmental potential of these embryos.

Despite the closeness of domestic cattle and gaur at the evolutionary level, major deficiencies result when applying reproductive technologies presumed most favourable for one species. Optimization of techniques for assisted reproduction requires a basic understanding of the species' endocrine, gamete and embryo/fetal parameters. The production of hybrids has been used extensively in research studies as they provide a unique opportunity to examine the fundamental differences between closely related species. For instance, minor differences between the gaur and domestic cattle genome resulted in gaur x cattle hybrids being used in genetic studies aimed at improving the resolution of the bovine gene map [20]. Dindot et al. [23] used cells from gaur x domestic cattle hybrids produced by artificial insemination as a model for investigating gene expression patterns after SCNT. The authors were able to show that SCNT a difference exists in the disturbance of gene expression between the TE and ICM, which causes disruption of genomic imprinting and hypermethylation in the chorion but not the fetus. Furthermore, hybridization of non-domestic and domestic cattle species has been of interest for the genetic improvement of farmed animals. Species such as the gaur can contribute resistance to heat, tolerance to disease and increased body weight to a domestic cattle herd. Interest in exploiting the advantages of heterosis has resulted in numerous attempts at understanding the species-specific nature of reproduction. The objectives of this study were to examine the effects of nuclear-cytoplasmic interactions on the developmental potential of interspecies IVF and SCNT embryos in an attempt to understand their role in SCNT embryo development. These objectives were undertaken by examining embryo parameters, including cell number and cell allocation, apoptosis index, gene expression profile and mtDNA content, to assess any alterations

resulting from the changing nuclear and mitochondrial environments. Gaur x domestic cattle IVF and SCNT hybrids were used as models to investigate the influence of the domestic cattle cytoplasm on different nuclear backgrounds. Information gathered from this study is hoped to help determine whether the production of gaur embryos using interspecies SCNT is a feasible alternative for the preservation of gaur genetic material.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Ethics

The present study was approved by the Animal Care Committees of the University of Guelph and the Toronto Zoo, approval number 99R147. The experiments were conducted in accordance with the requirements of the Animals for Research Act of Ontario, revised 1990, and the recommendations of the Canadian Council for Animal Care.

Experimental Design

Embryos were produced from domestic cattle and gaur in the following combinations (male x female IVF or donor cell x recipient oocyte SCNT): cattle x cattle IVF (cattle IVF), cattle x cattle SCNT (cattle NT), gaur x cattle IVF (gaur IVF), gaur x cattle SCNT (gaur NT), and hybrid x cattle SCNT (hybrid NT). All IVF and SCNT experiments were repeated 5 times and embryos for further analyses were randomly selected from each of the 5 runs. Embryos were evaluated for cleavage on day 2 and blastocyst development on day 8 (at 196 hours post insemination / activation). Cleavage rates were reported as a percentage of total presumptive zygotes placed in culture (number of cleaved embryos / total number of embryos) and blastocyst rates were reported as a percentage of cleaved embryos (number of blastocysts / number of

cleaved embryos). Morphology was evaluated and images were recorded at 40X magnification using a Leica inverted microscope.

Preparation of Fibroblast Cells

Domestic cattle (*Bos taurus*) samples from ear tissue collected post-mortem were obtained from slaughterhouse material. Gaur (*Bos gaurus*) samples from ear tissue collected post-mortem were obtained from gaur from the Toronto Zoo (Toronto, ON, Canada). The hybrid donor cells were collected from tissue biopsies from a gaur x cattle hybrid fetus produced by artificial insemination of domestic cows with gaur semen (cells provided by Dr. J. Piedrahita, North Carolina State University, Raleigh, NC, USA). Ear samples were prepared by digestion in 0.5% collagenase type I in Dulbecco Modified Eagle Medium (DMEM) for 5-6 hours at 38.5 °C and culture in DMEM + 1% Penicillin/Streptomycin (Invitrogen Canada Inc., Burlington, ON, Canada) + 10% FBS at 38.5°C in 5% CO_2 in air. Cytogenetic analysis was carried out on all cultures to ensure >80% chromosomally normal cells present. Cells were grown to passage 5 and maintained in confluence 2-3 days prior to use for SCNT.

Preparation of IVF Embryos

Domestic cattle oocytes were obtained from slaughterhouse ovaries and subjected to in vitro maturation (IVM), fertilization (IVF), and culture (IVC) as described in Mastromonaco et al. [24]. Briefly, cumulus-oocyte complexes (COCs) were matured in 80 µl drops of mSOFaa medium + 2% steer serum (IVM) supplemented with 1 µg/ml estradiol, 0.5 µg/ml FSH and 1 µg/ml LH (IVM + H) under silicone oil for 20-22 hours at 38.5°C in 5% CO_2 in air. Co-incubation with sperm was carried out in 80 µl drops of IVF TALP under silicone oil for 18 hours at 38.5°C in 5% CO_2 in air. Frozen-thawed sperm was prepared by percoll gradient (45%:90%, v:v) centrifugation at 700 x g for 30 minutes. The oocytes were inseminated with a

final concentration of 1 x 10^6 motile sperm/ml of either: a) frozen-thawed domestic cattle sperm (Gencor, Guelph, ON, Canada) or b) frozen-thawed gaur sperm. Embryos were cultured in 30 µl drops of mSOFaa medium + 8 mg/ml fatty acid-free BSA (IVC) under silicone oil at 38.5°C in 5% CO_2, 5% O_2, 90% N_2 for 8 days.

Preparation of SCNT Embryos

SCNT was carried out according to Mastromonaco et al. [24]. Cattle COCs were matured as described above. After 18 hours of maturation, cumulus cells were removed by vortexing in 1 mg/ml hyaluronidase for 2 minutes. Oocytes with polar bodies were stained with 5 µg/ml Hoechst 33342 for 2-3 minutes and enucleated mechanically. Manipulations were carried out in 40 µl drops of modified Tyrode medium under silicone oil. Fusion of the cytoplast and donor cell was accomplished in 0.28M mannitol containing 100 µM $MgCl_2$ and $CaCl_2$ with an electrical stimulus of 1.5 kV/cm for 40 µs. The reconstructed embryos were activated for 5 minutes in 5 uM ionomycin followed by 5 hours in 10µg/ml cycloheximide. Embryos were cultured in mSOFaa medium as described above.

Differential Staining of Blastocysts

Blastocysts collected on day 8 of culture at 196 hours were used. The staining was done using propidium iodide and Hoechst 33342 according to the protocol of Thouas et al. [25]. Differentially stained cell lineages were analyzed and images were recorded at 40X magnification using a Leitz fluorescence microscope. Trophectoderm (TE) cells were stained pink and inner cell mass (ICM) cells were stained blue.

TUNEL Staining of Embryos

Early cleavage stage embryos to be analyzed were collected at 45 hours (4-8 cell), 80 hours (9-16 cell) and 196 hours (blastocyst) of culture. Following fixation in 4%

paraformaldehyde for 1 hour, the embryos were stored in 1% paraformaldehyde at 4°C for up to 2 weeks. TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling) assay (In Situ Cell Death Detection Kit, Roche Diagnostics, Indianapolis, IN, USA) was carried out according to the method of Gjorret et al. [26]. TUNEL staining was analyzed and images were recorded at 40X magnification using a Leitz fluorescence microscope. All nuclei were stained red and TUNEL-positive nuclei were yellow-green. Changes in nuclear morphology (condensed red nuclei) accompanied by DNA fragmentation (condensed yellow-green nuclei) were assessed and defined as apoptotic morphology (M+T). Apoptotic indices were reported as the percentage of total embryos (number of embryos containing at least one cell displaying M+T / total number of embryos). Affected blastomeres were reported as a percentage of total blastomeres (number of blastomeres in the embryo displaying M+T / total number of blastomeres in the embryo). Within the blastocyst, detection of apoptotic blastomeres is described in putative ICM and TE cells as true cell allocation can only be confirmed by differential staining.

Gene Expression Profiles of Blastocysts

The genes of interest belonged to two categories: nuclear-encoded and mitochondrial-encoded. The three nuclear-encoded genes included: nuclear respiratory factor 2 (*NFE2L2*: transcriptional regulator for genes encoding the subunits of the oxidative phosphorylation (OXPHOS) system and genes involved in mtDNA replication), and the pro-survival *BCL2* and pro-apoptotic *BAX* (genes involved in the apoptotic pathway). The two mitochondrial-encoded genes consisted of: Cytochrome b and Cytochrome c Oxidase II (*MT-CYB*, *MT-CO2*: genes encoding subunits of the OXPHOS system).

mRNA Extraction and cDNA Preparation

Blastocysts collected on day 8 of culture at 196 hours were washed in 0.1% PBS-PVA, snap-frozen in pools of 5 embryos in liquid nitrogen and stored at -80°C until ready for use. Extraction of mRNA was carried out using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA) followed by precipitation using 6 µl sodium acetate (Ambion, Austin TX, USA), 1 µl linear acrylamide (Ambion) and 60 µl chilled isopropanol (Fisher Scientific). The air-dried pellet was resuspended in 4 µl of RNase-free water (Invitrogen Canada Inc.) and the solution was reverse transcribed to cDNA using standard RT-PCR protocols. The resulting cDNA was stored at –20°C until ready to be used for Real Time PCR analysis.

Primer Design

Complementary DNAs of interest were amplified using specific oligonucleotide primers for *BCL2, BAX, NFE2L2, MT-CYB, MT-CO2* and the housekeeping gene β-Actin (*ACTB* described in the bovine embryo by Favetta et al. [27]) as listed in Table 1. To confirm the specificity of the primers, the gene products were amplified using bovine liver RT reactions, which were performed on 5 µg of total RNA using standard procedure. A cDNA aliquot of 1 µl was subjected to PCR amplification in the PTC 200 Thermocycler (MJ Research). Each product underwent the following program of amplification: activation at 94°C for 5 minutes followed by a number of amplification cycles dependent on the gene (Table 2), including: denaturation at 94°C for 1 minute, primer annealing for 1 minute (Table 2) and elongation at 72°C for 1 minute, followed by a final elongation step at 72°C for 10 minutes. Aliquots of the PCR products were separated on a 2% agarose gel. The specific band for each gene was extracted using Qiaquick gel extraction columns (Qiagen Inc.). The purified PCR products were quantified along with a low DNA mass ladder (Invitrogen Canada Inc.) and 20 ng of each product (10 ng/µl) were sent to be sequenced (Univeristy of Guelph Molecular Supercentre, Guelph, ON, Canada) to confirm their

identity and were further used to calibrate the Real Time PCR assay.

Absolute Quantification of mRNA Levels

Purified and quantified PCR products were diluted for the Real Time PCR standard curves. The Real Time PCRs were conducted in a Light Cycler (Roche Diagnostics) and the products were detected using the FastStart Master SYBR Green I mix (Roche Diagnostics) following the protocol described by Favetta et al. [27]. For each quantification a 1 μl aliquot of the RT reaction was used according to the specific conditions for each set of primers (Table 2). The amplification program was as follows: preincubation for FastStart polymerase activation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C (20°C/second), annealing for 5 sec (20°C/second) at a gene-specific temperature (Table 2), elongation at 72°C for a gene-specific amount of time (Table 2) and acquisition of fluorescence for 5 seconds (20°C/second) at a gene-specific temperature (Table 2). The melting curve of the amplified product was achieved by initiating fluorescence at 72°C and taking measurements every 0.1°C until 95°C. Amplification was performed using β-Actin as an endogenous standard to ensure equal RT efficiency. Each reaction was performed in triplicates on three different pools of samples consisting of 5 blastocyst stage embryos. Total amount of mRNA per pooled sample was converted to amount of mRNA per cell (amount of mRNA / average total cell number) to avoid discrepancies due to differences in blastocyst cell numbers among the treatment groups. Levels of mRNA in cattle NT, gaur IVF, gaur NT and hybrid NT groups were expressed as relative abundance to cattle IVF mRNA levels.

Mitochondrial DNA Analysis of Gaur NT Embryos

Isolation of Genomic DNA

Gaur NT embryos were collected at various stages from post-fusion to day 8 of culture

including: 1 cell, 2 cell, 4 cell, 8 cell, 16 cell, morula and blastocyst stages. The embryos were washed in 0.1% PBS-PVA, snap-frozen individually in liquid nitrogen and stored at -80°C until ready for use. Genomic DNA was isolated from individual embryos using the QIAamp DNA Microkit (Qiagen Inc., Mississauga, ON, Canada). Carrier DNA (500 ng) was added to each sample before extraction and the recovered DNA was resuspended in 50 µl of DMPC H_2O and diluted 5 times before quantification.

Absolute Quantification of Mitochondrial DNA Copy Number

Primer pairs for the absolute quantification of mtDNA copy number are listed below:

MT12Sint2-F: 5'-AGGAGTACGGCGTAAAACGT-3'

MT12Sint2-R: 5'-GCATAGTGGGGTATCTAATC-5'

GAURSP-F: 5'CCTGATTTAAACTATTTCCTAA-3'

GAURSP-R: 5'ATTCTGTAGTGGTGCGGGA-3'

Quantification standards were prepared according to the protocol of Thundathil et al. [28]. A 1211 bp fragment of the 12S region of mtDNA was amplified and the PCR product was extracted from the gel using QIAquick Gel Extraction Kit (Qiagen Inc.) and cloned in the pGMT plasmid (Promega – Fisher Scientific, Nepean, ON, Canada). The plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen Inc.), quantified by spectrophotometer and diluted at 1×10^7 copies/µl in 20 µl aliquots and frozen. The polymorphic region of interest included the 17 bp addition to the 5' end of the d-loop in the gaur (124 bp fragment in gaur mtDNA corresponded to a 107 bp fragment in domestic cattle (taurus) mtDNA). To simulate embryo samples, standard stock was extracted and serially diluted for use in the standard curve. Real Time PCR was performed in a LightCycler (Roche Diagnostics) as described by Thundathil et al. (2005) with modifications. Premix for quantitative PCR was prepared from LightCycler FastStart

DNA Master SYBR Green I (Roche Diagnostics). For each quantification a 10 µl aliquot of DNA template was used. The PCR program employed an initial step of 95°C for 5 minutes followed by 45 cycles of 5 seconds each at 94°C for denaturation, 4 seconds at 57°C for annealing, and 9 seconds at 72°C for elongation. The melting curve of the amplified product was achieved at 95°C for 5 seconds, 55°C for 5 seconds, and 99°C for 0 seconds. Cooling was done at 40°C for 10 seconds. Temperature transition rates were set for 20°C/second except for the final melting step (0.1°C/second). Fluorescent signal was acquired at the end of elongation for quantification and the final step of continuous melting for identification of the PCR product. Each reaction was performed on individual embryos.

Statistical Analysis

In all experiments N represents the total number of presumptive zygotes placed in culture. Values are reported as means ± the standard error of the mean (SEM). The effect of the treatments was analyzed using the one-way ANOVA, followed by Tukey's post-hoc analysis or the equivalent non-parametrical test (Kruskal-Wallis test) when samples did not meet the assumption of normal distribution or homogeneity of variance and using the Chi-square test for percentages (Minitab for Windows, Minitab Inc., 1998). Differences with probabilities (p) < 0.05 were considered significant.

RESULTS

Development of Cattle and Hybrid Embryos

To examine the effects of the interaction between the gaur nucleus and domestic cattle cytoplasm on embryo development, the following treatment groups were assessed: cattle IVF (100% cattle nucleus), cattle NT (100% cattle nucleus), gaur IVF (50% gaur nucleus), gaur NT (100% gaur nucleus) and hybrid NT (50% gaur nucleus). Differences were observed in the

cleavage rates between treatment groups (Table 3). Cattle NT and gaur NT cleavages were significantly lower than that of the cattle IVF group, and the gaur NT cleavage frequency was significantly lower than gaur IVF. This is most likely not due to the intrinsic qualities of the embryos themselves but due to technical differences between the IVF and SCNT techniques. Difficulty in identifying lysed donor cells following fusion and, on several occasions, extrusion of the donor cell into the perivitelline space following oocyte activation may result in the inclusion of improperly reconstructed embryos. Thus, to eliminate these discrepancies, blastocyst development rates are presented as a percentage of cleaved embryos rather than total embryos.

Embryos reaching the blastocyst stage by days 7 and 8 of culture varied between certain treatment groups (Table 3). No differences were observed between cattle IVF and NT groups. The proportion of blastocysts on day 7 was comparable for all groups except for gaur NT, which was significantly lower. Development of gaur NT embryos on day 8 was again significantly lower than all other groups. In contrast, improved blastocyst rates were observed in the hybrid NT group on both day 7 and 8, comparable to those of cattle IVF and gaur IVF groups.

Embryo quality, based on morphological observations under light microscopy, was similar among cattle IVF and NT embryos, which developed into large expanded blastocysts with a well-defined inner cell mass (figure not shown; described in Mastromonaco et al. [24]). Gaur IVF and hybrid NT embryos exhibited similar morphology to the cattle groups, forming expanded blastocysts in a timely manner, but not as large as those of cattle embryos. Gaur NT embryos appeared as good quality, morphologically normal (equal sized blastomeres, no fragmentation) embryos until approximately the 8-16 cell stage (Figure 1A-A'). Compacted morulae showed signs of fragmentation and degeneration (Figure 1B-B'). Delayed development was observed after day 6 as the majority of day 7 embryos consisted of compact morulae or early

blastocysts in the process of forming the blastocoel (Figure 1C-C'). Day 8 blastocysts consisted mainly of blastocysts beginning to expand (Figure 1D-D'). The inner cell mass was not compact and well-defined, instead, it appeared to spread out over the embryo surface.

Blastocyst quality was also assessed quantitatively. Differentially stained blastocysts were evaluated for the total number of cells and the number of cells allocated to the ICM and TE on day 8 (Table 4). The total cell number of blastocysts from the cattle treatment groups ranged between 185-195 cells. Embryos from both the hybrid NT and gaur IVF groups showed similar growth in cell number. In contrast, gaur NT blastocysts had significantly lower cell numbers, averaging 100 cells. The decrease was observed in both cell types, TE and ICM, compared to the other treatment groups. Despite the striking difference in total cell number, the ratio of ICM cells to total cells was similar in all treatment groups (Table 4).

Incidence of Apoptosis in Cattle and Hybrid Embryos

Examination of apoptotic morphology at early cleavage and blastocyst stages was performed (Figure 2). The percentage of embryos containing at least one cell displaying apoptotic morphology increased during the developmental stages examined, with all embryos being affected by the blastocyst stage (Figure 3). This was similar for all treatment groups. Cattle IVF embryos did not show signs of apoptotic morphology until the 9-16 cell stage unlike cattle NT embryos which had a greater percentage of apoptotic embryos first noted at the 4-8 cell stage. The gaur NT group displayed a similar level of apoptotic morphology at the 9-16 cell stage as cattle NT however, no evidence was observed at the 4-8 cell stage. Interestingly, hybrid NT embryos did not contain any cells with apoptotic morphology in the early cleavage stages. A significantly greater incidence was only observed in the gaur IVF group in both 4-8 and 9-16 cell embryos. To better explain variations in apoptotic indices among the treatment groups, the

number of blastomeres per embryo showing signs of apoptotic morphology was examined. In the early cleavage stage embryos, a significantly higher percentage of blastomeres with apoptotic morphology was observed in the gaur IVF group, whereas no remarkable differences occurred in any of the other groups (Figure 4). At the blastocyst stage, although a tendency existed for the ICM to have a greater number of abnormal blastomeres, particularly in the gaur IVF, gaur NT and hybrid NT groups, no significant differences were detected (Figure 4).

Expression of Mitochondria-related Genes in Cattle and Hybrid Embryos

The expression profiles of several mitochondria-related genes were examined at the blastocyst stage. Differences in the relative abundance of mRNA levels (compared to cattle IVF levels) were observed among the treatment groups. *NFE2L2, MT-CYB* and *MT-CO2* levels were similar in the cattle NT and gaur NT groups, both of which were significantly greater than the other groups (Figure 5A). Cattle IVF, gaur IVF and hybrid NT embryos exhibited similar mRNA profiles with the exception of the increased *MT-CO2* levels in the gaur IVF group. Expression of *BCL2* mRNA was significantly lower in gaur NT embryos than other treatment groups (Figure 5B). Hybrid NT *BCL2* mRNA levels were intermediate between gaur NT and the remaining embryo groups. Levels of *BAX* mRNA were also significantly decreased in gaur NT and hybrid NT embryos. No significant differences were observed in the ratio of *BCL2*: *BAX* , however, SCNT embryos tended to have decreased levels of *BCL2*: *BAX* , with the lowest being observed in the gaur NT group.

mtDNA Content of Gaur NT Embryos

In the gaur NT embryos, the number of copies of mtDNA and their origin were examined. The total number of mtDNA copies per embryo ranged from approximately 250,000 – 750,000 (Figure 6A). This is consistent with numbers previously documented in cattle embryos

[29]. The number of mtDNA copies remained constant during the early cleavage stages until the blastocyst stage, at which point a significant increase was detected. Similar results were observed for the number of gaur mtDNA copies per embryo (Figure 6B). The copies of gaur mtDNA per embryo were shown to increase from approximately 600 in the recently reconstructed embryo to 2000 in the blastocyst. The ratio of gaur: total mtDNA, denoting the contribution of gaur mitochondria to the pool of domestic cattle mitochondria within the cytoplasm, averaged 0.1% in the newly reconstructed embryos (1-2 cell stage) (Figure 6C). This remained relatively constant until the blastocyst stage when a significant increase in the proportion of gaur mtDNA was observed. The significant increase was detected in 4 of the 12 (33%) blastocysts analyzed.

DISCUSSION

The results of this study clearly demonstrate that the nuclear-cytoplasmic environment in interspecies SCNT embryos has an effect on the developmental potential of reconstructed embryos. Both the quantity and quality of embryos were affected by the make-up of the nuclear genome directing cellular processes past the stage of embryonic genome activation. Striking differences observed between SCNT embryos consisting of a 100% gaur nuclear genome and a 50% gaur: 50% cattle nuclear genome within a cattle cytoplasmic environment provide evidence for the role of nuclear-cytoplasmic interactions in embryo developmental competence. Although gaur SCNT embryo production using domestic cattle oocytes has been attempted and pregnancies were established [2], a better understanding of the compatibility of these closely related species is necessary to explain the decreased developmental capacity of these embryos.

It is reasonable to assume that increased chances of successful interspecies in vitro embryo production exist if the species involved hybridize naturally as in the gaur and domestic

cattle. In spite of this, differences in day 7 and 8 blastocyst rates among the treatment groups in our study provide evidence to indicate that the nuclear genome constitution of the gaur-cattle hybrid embryos has an effect on their developmental potential. Firstly, it is important to note the similarities between cattle IVF and cattle NT embryo development.We have therefore excluded technical factors resulting from our SCNT protocol as a cause of the observed differences among the hybrid treatment groups. Secondly, the percentages of gaur NT and hybrid NT embryo development were similar to or greater than previously reported [2, 23]. Thus, the dramatic reduction in blastocyst numbers on day 7/8 of culture in the gaur NT group suggests that a gaur nucleus within a domestic cattle cytoplasmic environment may not be properly capable of directing embryo development in the later preimplantation stages. Furthermore, delayed or arrested development and decreased embryo quality following the production of good quality, morphologically normal 8-16 cell stage embryos, as evidenced by the numbers of morulae still present on day 7 and the higher percentage of fragmented morulae and blastocysts with poorly-defined ICM, confirm that problems arise at the time when the gaur genome assumes control.

The importance of adequate nuclear-cytoplasmic compatibility for normal development is substantiated by the fact that gaur IVF and hybrid NT embryo development were similar to the cattle controls. In both these cases, the gaur constituted only 50% of the nuclear genome, while the cytoplasm was 100% cattle, unlike the gaur NT embryos, which were found to contain primarily cattle mtDNA (>99%) in a 100% gaur nuclear background. Thus, as described by Hiendleder et al. [30], when the cattle mitochondrial genes are complemented by a corresponding set of maternal nuclear genes, some nuclear-cytoplasmic compatibility exists between the two species. The same could be said for the possible recognition of other organelles and cytoplasmic elements by the corresponding nuclear genes. This scenario is exactly what

occurs during natural hybridization, which may explain the ability to obtain live offspring from gaur-domestic cattle crosses. In addition, the fact that gaur IVF and hybrid NT blastocyst rates were similar suggests that reprogramming of the donor nucleus following SCNT may not be a major factor contributing to the poor developmental outcome of gaur NT embryos, thereby emphasizing the possible role of nuclear-cytoplasmic communication.Reports in SCNT embryos to date indicate that both heteroplasmic and homoplasmic embryos result from this technique due to preferential or lack of replication of the donor mitochondria [31-33]. The complexity of nuclear-mitochondrial communication would suggest that both of these situations are disadvantageous for normal embryo growth, with alterations in metabolic, apoptotic and physiologic profiles being expected. Interestingly, a degree of preferential replication of the gaur mtDNA was observed in the gaur NT embryos at the blastocyst stage. However, not all blastocysts were replicating the gaur mtDNA. The significant increase was observed in only 30% of the blastocysts, with the remaining ones showing passive transmission of the gaur mtDNA. This is similar to the results obtained in the *Bos indicus – Bos taurus* model used by Meirelles et al. [32]. The questions to consider are whether the few blastocysts selectively replicating their own mtDNA are more competent than the others and whether the gaur mtDNA would continue to increase or be eliminated by the fetal and neonatal stages.

Numerous studies have correlated poor embryo quality and quantity with alterations in cellular parameters such as chromosome content, metabolism, apoptosis and gene expression. Timing and quality of blastocyst development are directly related to total cell number and allocation of the cell lineages [34-35]. IVF [36] and SCNT [37] embryos displayed aberrant cell allocation compared to in vivo produced embryos, In our study, no differences in total cell number and cell allocation were observed between cattle IVF and SCNT embryos. However,

gaur NT embryos did exhibit a significantly lower total number of cells at the blastocyst stage,clearly visible by morphological assessment.. Low cell numbers may indicate that the poor quality and delayed development of the gaur NT embryos are due to alterations in cell division. Several studies have correlated delayed or arrested cell division with mitochondrial dysfunction and a decreased capacity for ATP generation [38-39]. These embryos displayed arrested or delayed development with lower cell numbers at specific developmental stages compared to normal embryos. Similarly, human xenomitochondrial cell hybrids exhibited reduced cell growth due to the impairment of respiratory complex I activity resulting from the inability of the foreign nucleus to restore mitochondrial respiratory function [16, 17]. These studies provide support of our assumption that the detrimental outcome in gaur NT embryos is related to incompatibilities between nuclear and cytoplasmic components, with particular reference to the mitochondria. In confirmation, adequate growth and cell number in hybrid NT, as well as gaur IVF, embryos may be the result of altered nuclear-cytoplasmic communication due to the presence of cattle genes. Interestingly, the ratio of ICM cells to total cells was not altered in any of the groups indicating that cell allocation was not affected in the poorly developing gaur NT embryos. Fischer-Brown et al. [40] suggest that the fundamental mechanism involved in cell allocation could be maintained despite differences in the rates of cell division.

Another mechanism that may have contributed to the decreased cell number in the gaur NT embryos is apoptosis. Normal blastocyst development requires adequate levels of apoptosis to eliminate damaged, defective or unwanted cells [41]. Excessive or inadequate levels of apoptosis may cause developmental retardation and/or embryo death. Thus, the incidence of apoptosis in embryos has been used as a measure of developmental potential. Compared to in vivo produced embryos, IVF and SCNT embryos generally display altered levels and timing of

apoptosis with a higher incidence and an earlier occurrence of apoptosis (in vivo < IVF < SCNT) [26, 42-43]. In this study, a similar trend in the incidence of apoptosis during the early cleavage stages, characterized by nuclear condensation/fragmentation and DNA fragmentation (M+T), was observed between the cattle IVF and cattle NT groups, although the differences were not statistically significant. Significantly greater levels in the incidence of apoptotic morphology and in the percentage of affected blastomeres were only detected in the early cleavage stages of gaur IVF embryos. This may be due to the fact that gaur x cattle hybrid embryos have an odd chromosome number (2n=59) and problems involving the gaur Rob(2;28) chromosome during post-fertilization mitoses may initiate apoptosis. It has been suggested that one of the possible roles for apoptosis might be the removal of chromosomally abnormal cells [41]. On the other hand, hybrid NT embryos (also 2n=59) did not display elevated levels of apoptosis.

As in previous reports of cattle embryos [26, 42-43], all the blastocysts produced in the various treatment groups had some degree of apoptosis. Studies have demonstrated that a higher percentage of apoptotic cells in the blastocyst were located in the ICM [26, 43]. Our results show similar trends but , no significant differences were found. Previous investigators had suggested that reduced blastocyst cell numbers following SCNT might be associated with the increased levels of apoptosis observed in those embryos [26, 43]. As no increase in apoptotic incidence was observed in gaur NT blastocysts, apoptosis per se does not appear to be the cause of the dramatic decrease in cell numbers. Other factors, possibly cell cycle related as discussed above, must be influencing the developmental outcome of the gaur NT embryos.

The apoptotic pathway is dependent on mitochondria, which play an active role in the regulation of the apoptotic cascade [44]. Thus, alterations in the levels of two genes (*BCL2*, pro-survival or ***BAX***, pro-death) have been associated with increased fragmentation and decreased

oocyte/embryo quality [45-46]. It has been suggested that the relative levels of expression of these genes can be used as an indicator of embryo quality, with an increase in *BCL2*: ***BAX*** ratio being associated with good embryo quality [46]. In the present study, the decreased ratio of *BCL2*: ***BAX*** in gaur NT embryos is indicative of poor developmental outcome and morphological appearance, such as the increase in fragmentation at the morula stage, of these embryos. Although no significant differences were reported in the ratio of *BCL2*: ***BAX*** , all SCNT groups had a tendency for decreased levels of *BCL2*: ***BAX*** , suggestive of lower developmental competence in SCNT embryos. This confirms numerous other reports, which show that SCNT embryos are compromised.

Jurisicova et al. [45] noted that a decrease in *BCL2*, the pro-survival gene, corresponded with an increase in the incidence of apoptosis and embryo fragmentation. Our observations did not conform to these findings. We noted that decreased *BCL2* expression is accompanied by low levels of apoptosis in the gaur NT group. Lack of proper communication between the nuclear-encoded *BCL2* of gaur origin and the mitochondrial components of the apoptotic pathway, mainly of cattle origin, may be the possible cause of the surprisingly low incidence of apoptosis in these poor quality embryos. Studies on humans have documented that one of the consequences of dysfunctional mitochondria was the dysregulation of apoptosis and subsequent disease [47]. Thus, the apoptotic profiles exhibited by the gaur NT embryos in the present study may be a sign of poor mitochondrial function resulting from inadequate communication between the nuclear and mitochondrial control elements rather than lack of apoptosis-inducing stimulation.

Altered gene expression patterns associated with SCNT embryos have been implicated as a possible cause of their poor developmental outcome [48-49]. Along with the apoptosis-related genes discussed above, *NFE2L2* (a nuclear-encoded transcriptional regulator of genes encoding

the subunits of the OXPHOS system and genes involved in mtDNA replication), *MT-CYB* and *MT-CO2* (both mitochondrial-encoded genes encoding subunits of the respiratory machinery) were assessed as well.*NFE2L2, MT-CYB* and *MT-CO2* mRNA levels were significantly elevated in both the cattle NT and gaur NT embryos. These observations suggest that there may be some dysregulation of gene expression related to the SCNT technique and not specific to the nuclear-cytoplasmic incompatibility in the interspecies embryos.

The gaur NT embryos produced consisted of the gaur genome directing the domestic cattle mitochondrial genome. Evidence gathered in this study indicates that xenomitochondrial homoplasmy results in developmentally compromised embryos. Delayed development and the corresponding decrease in cell number, as well as the decreased ***BCL2***: *BAX* ratio without a parallel increase in apoptotic incidence, in the gaur NT embryos are all signs of disrupted cellular processes. These parameters are correlated with mitochondrial function and it is known that any dysregulation in mitochondrial biogenesis or function results in decreased ATP levels and subsequent disruption of cellular processes. Analysis of gaur IVF and hybrid NT embryos confirms that developmental potential is improved when a portion of the nuclear genome corresponds to the inherited mitochondria, thereby assuring some interaction between the two genomes. Thus, poor or non-existent communication between the gaur nucleus and cattle cytoplasm may be a likely source of the detrimental features observed following gaur x cattle SCNT. At this time however, we cannot discount the effect that other cytoplasmic elements may have on interspecies compatibility. Further studies are necessary to examine the specific mechanisms that are disrupted, such as mitochondrial differentiation and organization during embryo development and the subsequent changes in respiratory activity and ATP production, as well as the contribution of other cytoplasmic components to cell cycle regulation, including the

centrosome and cytoskeletal network. Improved developmental competence of interspecific embryos may be achieved with the introduction of large numbers of donor mitochondria or other donor organelles of unknown significance at this time.

Interspecies SCNT is a valuable tool, not only as an alternative method for the preservation and propagation of non-domestic and endangered species, but as a means for studying the fundamental properties of embryo development. The evidence to date suggests that careful consideration of the evolutionary relationships between species , along with a better understanding of the mechanisms involved in nuclear-cytoplasmic communication, will help overcome the obstacles currently faced with interspecies SCNT.

Acknowledgements

The authors wish to thank Dr. Graham Crawshaw and the animal care staff at Toronto Zoo for their assistance with the gaur sample collection; Dr. Claire Plante for her assistance with the gaur semen collection and cryopreservation; Dr. Jorge Piedrahita for providing the hybrid cells; Esther Semple, Ed Reyes, Liz St. John, Grant Perry, and Raj Kukreja for their technical assistance.

References

1. White KL, Bunch TD, Mitalipov S, Reed WA. Establishment of pregnancy after the transfer of nuclear transfer embryos produced from the fusion of Argali (*Ovis ammon*) nuclei into domestic sheep (*Ovis aries*) enucleated oocytes. Cloning 1999; 1:47-54.
2. Lanza RP, Cibelli JB, Diaz F, Moraes CT, Farin PW, Farin CE, Hammer CJ, West MD, Damiani P. Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. Cloning 2000; 2:79-90.
3. Gomez MC, Jenkins JA, Giraldo A, Harris RF, King A, Dresser BL, Pope CE. Nuclear

transfer of synchronized african wild cat somatic cells into enucleated domestic cat oocytes. Biol Reprod 2003; 69:1032-1041.

4. Sansinena M, Hylan D, Hebert K, Denniston RS, Godke RA. Banteng (*Bos javanicus*) embryos and pregnancies produced by interspecies nuclear transfer. Theriogenology 2005; 63:1081-1091.

5. Dominko T, Mitalipova M, Haley B, Beyhan Z, Memili E, McKusick B, First NL. Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. Biol Reprod 1999; 60:1496-1502.

6. Kitiyanant Y, Saikhun J, Chaisalee B, White KL, Pavasuthipaisit K. Somatic cell cloning in buffalo (*Bubalus bubalis*): effects of interspecies cytoplasmic recipients, activation procedures. Cloning, Stem Cells 2001; 3:97-104.

7. Shin MR, Park SW, Shim H, Kim NH. Nuclear and microtubule reorganization in nuclear-transferred bovine embryos. Mol Reprod Dev 2002; 62:74-82.

8. Sankaran S, Parvin JD. Centrosome function in normal and tumor cells. J Cell Biochem 2006; 99:1240-1250.

9. Dai Y, Wang L, Wang H, Lui Y, Li N, Lyu Q, Keefe DL, Albertini DF, Liu L. Fate of centrosomes following somatic cell nuclear transfer (SCNT) in bovine embryos. Reproduction 2006; 131:1051-1061.

10. Ruiz-Pesini E, Diez C, Lapena AC, Perez-Martos A, Montoya J, Alvarez E, Arenas J, Lopez-Perez MJ. Correlation of sperm motility with mitochondrial enzymatic activities. Clin Chem 1998; 44:1616-1620.

11. Van Blerkom J, Davis PW, Lee J. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. Hum Reprod 1995;

530 10:415-424.

12. Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Goncalves PB, Wolf E. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. Biol Reprod 2001; 64:904-909.

535 13. Reynier R, May-Panloup P, Chretien M-F, Morgan CJ, Jean M, Savagner F, Barriere P, Malthiery Y. Mitochondrial DNA content affects the fertilizability of human oocytes. Mol Hum Reprod 2001; 7:425-429.

14. Hiendleder S, Wolf E. The mitochondrial genome in embryo technologies. Reprod Dom 540 Anim 2003; 38:290-304.

15. Hiendleder S, Prelle K, Bruggerhoff K, Reichenbach H, Wenigerkind H, Bebbere D, Stojkovic M, Muller S, Brem G, Zakhartchenko V, Wolf E. Nuclear-cytoplasmic interactions affect in utero developmental capacity, phenotype, and cellular metabolism of bovine nuclear transfer fetuses. Biol Reprod 2004; 70:1196-1205.

545 16. Kenyon L, Moraes CT. Expanding the functional human mitochondrial DNA database by the establishment of primate xenomitochondrial cybrids. PNAS 1997; 94:9131-9135.

17. Barrientos A, Kenyon L, Moraes CT. Human xenomitochondrial cybrids. Cellular models of mitochondrial complex I deficiency. J Biol Chem 1998; 273:14210-14217.

18. Dey R, Barrientos A, Moraes CT. Functional constraints of nuclear-mitochondrial DNA 550 interactions in xenomitochondrial rodent cell lines. J Biol Chem 2000; 275:31520-31527.

19. Buntjer JB, Otsen M, Nijman IJ, Kuiper MTR and Lenstra JA. Phylogeny of bovine species

based on AFLP fingerprinting. Heredity 2002; 88:46-51.

20. Riggs PK, Owens KE, Rexroad CE III, Amaral MEJ, Womack JE. Development and initial characterization of a *Bos taurus* x *B. gaurus* interspecific hybrid backcross panel. J Hered 1997; 88:373-379.

21. Winter H, Mayr B, Schleger W, Dworak E, Krutzler J, Burger B. Karyotyping, red blood cell and haemoglobin typing of the mithun (*Bos frontalis*), its wild ancestor and its hybrids. Res Vet Sci 1984; 36:276-283.

22. Bongso TA, Hilmi M, Sopian M, Zulkifli S. Chromosomes of gaur cross domestic cattle hybrids. Res Vet Sci 1988; 44:251-254.

23. Dindot SV, Farin PW, Farin CE, Romano J, Walker S, Long C, Piedrahita JA. Epigenetic and genomic imprinting analysis in nuclear transfer derived *Bos gaurus* / *Bos taurus* hybrid fetuses. Biol Reprod 2004; 71:470-478.

24. Mastromonaco GF, Semple E, Robert C, Rho J, Betts DH, King WA. Different culture media requirements of IVF and nuclear transfer bovine embryos. Reprod Dom Anim 2004; 39:462-467.

25. Thouas GA, Korfiatis NA, French AJ, Jones GM, Trounson AO. Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reprod Biomed Online 2001; 3:25-29.

26. Gjorret JO, Knijn HM, Dieleman SJ, Avery B, Larsson L, Maddox-Hyttel P. Chronology of apoptosis in bovine embryos produced in vivo and in vitro. Biol Reprod 2003; 69:1193-1200.

27. Favetta LA, Robert C, King WA, Betts DH. Expression profiles of p53 and p66shc during oxidative stress-induced senescence in fetal bovine fibroblasts. Exp Cell Res 2004; 299:36-48.

28. Thundathil J, Filion F, Smith LC. Molecular control of mitochondrial function in preimplantation mouse embryos. Mol Reprod Dev 2005; 71:405-413.

29. Smith LC, Thundathil J, Filion F. Role of the mitochondrial genome in preimplantation development and assisted reproductive technologies. Reprod Fertil Dev 2005; 17:15-22.

30. Hiendleder S, Zakhartchenko V, Wolf E. Mitochondria and the success of somatic cell nuclear transfer cloning: from nuclear-mitochondrial interactions to mitochondrial complementation and mitochondrial DNA recombination. Reprod Fertil Dev 2005; 17:69-83.

31. Steinborn R, Schinogl P, Wells DN, Bergthaler A, Muller M, Brem G. Coexistence of *Bos taurus* and *Bos indicus* mitochondrial DNAs in nuclear transfer-derived somatic cattle clones. Genetics, 2002; 162:823-829.

32. Meirelles FV, Bordignon V, Watanabe Y, Watanabe M, Dayan A, Lobo RB, Garcia JM, Smith LC. Complete replacement of the mitochondrial genotype of a Bos indicus calf reconstructed by nuclear transfer to a *Bos taurus* oocyte. Genetics 2001; 158:351-356.

33. Takeda K, Akagi S, Takahashi S, Onishi A, Hanada H, Pinkert CA. Mitochondrial activity in response to serum starvation in bovine (*Bos taurus*) cell culture. Cloning, Stem Cells 2002; 4:223-229.

34. Van Soom A, Vanroose G, de Kruif A. Blastocyst evaluation by means of differential staining: a practical approach. Reprod Dom Anim 2001; 36:29-35.

35. Van Soom A, Ysebaert MT, de Kruif A. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro produced bovine embryos. Mol Reprod Dev 1997; 47:47-56.

36. Van Soom A, Ysebaert MT, de Kruif A. Cell allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media. Mol Reprod Dev 1996; 45:171-82.

37. Koo D-B, Kang Y-K, Choi Y-H, Park JS, Kim H-N, Oh KB, Son D-S, Park H, Lee K-K, Han Y-M. Aberrant allocations of inner cell mass, trophectoderm cells in bovine nuclear transfer blastocysts. Biol Reprod 2002; 67: 487-492.

38. Van Blerkom J, Davis P, Alexander S. Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: relationship to microtubular organization, ATP content and competence. Hum Reprod 2000; 15:2621-2633.

39. Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. Reprod Fertil Dev 2005; 17:371-378.

40. Fischer-Brown A, Monson R, Parrish J, Rutledge J. Cell allocation in bovine embryos cultured in two media under two oxygen concentrations. Zygote 2002; 10:341-348.

41. Hardy K. Cell death in the mammalian blastocyst. Mol Hum Reprod 1997; 3:919-925.

42. Gjorret JO, Wengle J, Maddox-Hyttel P, King WA. Chronological appearance of apoptosis in bovine embryos reconstructed by somatic cell nuclear transfer from quiescent granulosa cells. Reprod Dom Anim 2005; 40:210-216.

43. Fahrudin M, Otoi T, Karja NWK, Mori M, Murakami M, Suzuki T. Analysis of DNA fragmentation in bovine somatic cell nuclear transfer embryos using TUNEL. Reproduction 2002; 124:813-819.

44. Garesse R, Vallejo CG. Animal mitochondrial biogenesis and function: a regulatory cross-talk between two genomes. Gene 2001; 263:1-16.

45. Jurisicova A, Latham KE, Casper RF, Varmuza SL. Expression and regulation of genes associated with cell death during murine preimplantation embryo development. Mol Reprod Dev 1998; 51:243-253.

46. Yang MY, Rajamahendran R. Expression of Bcl-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced in vitro. Anim Reprod Sci 2002; 70:159-169.

47. Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis, necrosis. Ann Rev Physiol 1998; 60:619-642.

48. Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R, Niemann H. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. Biol Reprod 2001; 65:309-317.

49. Daniels R, Hall V, Trounson AO. Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. Biol Reprod 2000; 63:1034-1040.

Figure Legends

Figure 1. Morphology of gaur NT embryos. Phase contrast micrographs depicting gaur NT embryos at various development stages: A-A') 8-12 cell stage; B-B') day 6 morula; C-C') day 7 morula / early blastocyst; D-D') day 8 blastocyst. A' to D' are magnified images of embryos in A to D. Images were recorded at 20X and 40X magnification.

Figure 2. Nuclear condensation / DNA fragmentation in intra- and interspecies IVF and SCNT embryos. Fluorescent micrographs demonstrating apoptotic morphology in IVF and SCNT embryos: A-B) Cattle IVF; C-D) Cattle NT; E-F) Gaur IVF; G-H) Gaur NT. Condensed red nuclei depict morphological signs without TUNEL labelling. Condensed yellow nuclei depict positive TUNEL labelling (M+T). Condensed red nuclei depict morphological signs without TUNEL labeling (M). In (A), note the presence of a polar body (PB) and sperm (SP). Images were recorded at 40X magnification.

Figure 3. Incidence of apoptotic morphology (M+T) in intra- and interspecies IVF and SCNT embryos. Developmental stages analyzed: 4-8 Cell; 9-16 Cell; Blastocyst. Numbers above the bars correspond to the number of embryos analyzed. Values are reported as means. (*,**) denotes a significant difference between treatments ($p<0.05$).

Figure 4. Percentage of blastomeres with apoptotic (M+T) morphology in intra- and interspecies IVF and SCNT embryos. Developmental stages analyzed: 4-8 Cell; 9-16 Cell; blastocyst inner cell mass (BL:ICM), blastocyst trophectoderm (BL:TE), blastocyst total cells (BL:Total). Values are reported as means ± the standard error of the mean (SEM). (*,**) denotes a significant difference between treatments ($p<0.05$).

Figure 5. Expression profiles of nuclear- and mitochondrial-encoded genes in intra- and interspecies IVF and SCNT embryos. mRNA expression levels relative to cattle IVF of: A)

nuclear-encoded *NFE2L2*and mitochondrial-encoded *MT-CYB* and *MT-CO2*; B) apoptosis-related *BCL2* and *BAX*. Values are reported as means ± the standard error of the mean (SEM). (^{a,b,c,d}) Superscripts denote a significant difference between treatments ($p<0.05$).

Figure 6. Mitochondrial DNA copy number in gaur NT embryos. Quantification of: A) total number of mtDNA copies per embryo; B) number of gaur mtDNA copies per embryo; C) ratio of gaur:taurus mtDNA per embryo. Developmental stages analyzed: 1-2 Cell; 4-8 Cell; 16 Cell – Morula; Blastocyst. Individual values are reported with the bars representing the means. ^{a,b} Superscripts denote a significant difference between developmental stages ($p<0.01$).

Table 1. Primers used for PCR.

Gene	GenBank Accession Number	Species	Sequence	Fragment Size (bp)
BCL2	U92434	*Bos Taurus*	5'-ATGACTTCTCTCGGCGCTAC-3' 5'-CGGTTCAGGTACTCGGTCAT-3'	244
BAX	U92569	*Bos Taurus*	5'-GCATCGGAGATGAATTGGAC-3' 5'-TGCCGTCAGAAAACATTTCA-3'	117
NFE2L2	AB162435	*Bos Taurus*	5'-TCCAACCTTTGTCGTCATCA-3' 5'-TTGCCCGTAGCTCATCTCTT-3'	174
MT-CYB	M28016	*Homo sapiens*	5'-AGGCGTCCTTGCCCTATTAC-3' 5'-CGGATGCTACTTGTCCAATG-3'	200
MT-CO2	X15759	*Homo sapiens*	5'-CCATCCCTACGCATCCTTTA-3' 5'-CGGGAATTGCATCTGTTTTT-3'	295
AXTB	BC008633	*Mus musculus*	5'-CGTGACATTAAGGAGAAGCTGTGC-3' 5'-CTCAGGAGGAGCAATGATCTTGAT-3'	374

Table 2. Conditions for PCR and Real Time PCR.

Gene	$MgCl_2$ Conc. (mM)	Annealing Temp. (°C)	Amplification Cycles	Primer Conc. Real Time (µM)	Fluorescence Acquisition Temp. (°C)	Elongation Time (sec) (2°C/sec)
BCL2	2	58	35	2.5	90	10
BAX	1.5	58	35	5	83	7
NFE2L2	2.5	57	35	2.5	83	7
MT-CYB	2.5	53	38	1	84	8
MT-CO2	3	52	40	2.5	83	12
ACTB	1.5	60	35	5	87	17

Table 3. Embryo development in intra- and interspecies IVF and SCNT embryos.

Embryo Production Method	Total Embryos (N)	Cleavage (%)	Day 7 Blastocyst (%)	Day 8 Blastocyst (%)
Cattle IVF	562	85.0 ± 0.7^{a}	19.1 ± 2.5^{a}	$35.7 \pm 1.4^{a,b}$
Cattle NT	145	$76.1 \pm 3.5^{b,c}$	26.2 ± 6.0^{a}	42.3 ± 1.6^{a}
Gaur IVF	562	$82.2 \pm 1.9^{a,b}$	18.8 ± 2.5^{a}	30.4 ± 1.5^{b}
Gaur NT	228	71.8 ± 4.0^{c}	1.5 ± 1.5^{b}	18.1 ± 2.9^{c}
Hybrid NT	191	$69.5 \pm 8.2^{a,b,c}$	13.2 ± 4.2^{a}	$32.5 \pm 3.7^{a,b}$

Values are reported as means ± the standard error of the mean (SEM). (^{a,b,c}) Superscripts within columns denote a significant difference between treatments ($p<0.05$).

Table 4. Cell number and allocation in intra- and interspecies IVF and SCNT embryos.

Embryo Production Method	Total Embryos (N)	ICM	TE	Total	ICM:Total
Cattle IVF	10	58.3 ± 5.7^{a}	134.8 ± 7.6^{a}	192.7 ± 11.2^{a}	0.30 ± 0.02^{a}
Cattle NT	10	62.2 ± 6.5^{a}	130.1 ± 13.6^{a}	192.3 ± 19.9^{a}	0.32 ± 0.01^{a}
Gaur IVF	10	$50.0 \pm 4.7^{a,b}$	135.5 ± 12.2^{a}	185.5 ± 14.1^{a}	0.27 ± 0.02^{a}
Gaur NT	10	34.7 ± 4.2^{b}	65.6 ± 4.6^{b}	100.3 ± 6.7^{b}	0.34 ± 0.03^{a}
Hybrid NT	10	63.1 ± 5.5^{a}	122.9 ± 8.8^{a}	186.0 ± 13.0^{a}	0.34 ± 0.02^{a}

ICM = inner cell mass cells; TE = trophectoderm cells; Total = total cells. Values are reported as means ± the standard error of the mean (SEM). ^{a,b} Superscripts within columns denote a significant difference between treatments ($p<0.05$).

Figure 1. Morphology of gaur NT embryos.

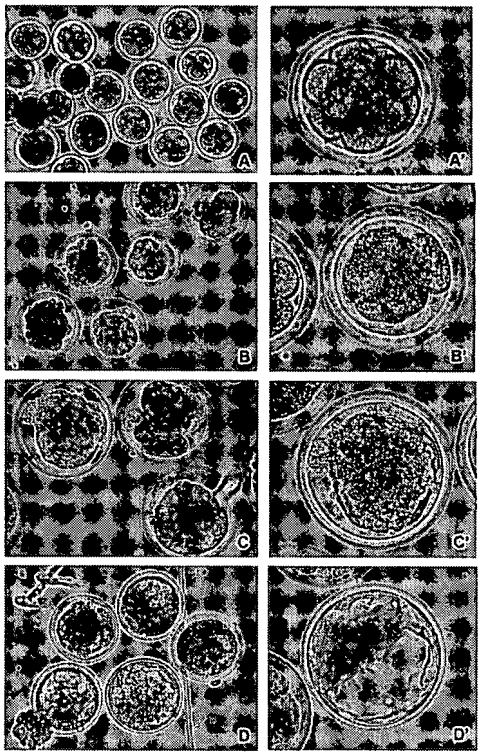

Figure 2. Nuclear condensation / DNA fragmentation in intra- and interspecies IVF and SCNT embryos.

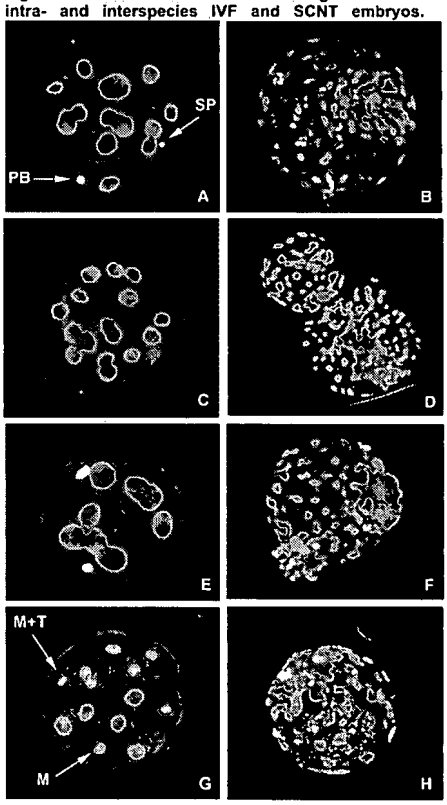

Figure 3. Incidence of apoptotic morphology (M+T) in intra- and interspecies IVF and SCNT embryos.

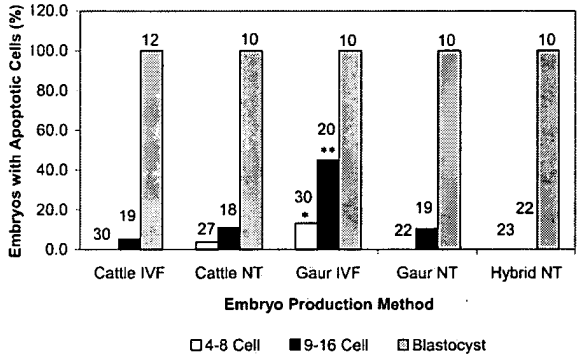

Figure 4. Percentage of blastomeres with apoptotic (M+T) morphology in intra- and interspecies IVF and SCNT embryos.

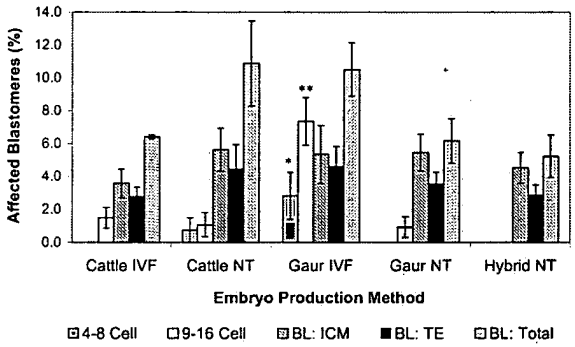

Figure 5. Expression profiles of nuclear- and mitochondrial-encoded genes in intra- and interspecies IVF and SCNT embryos.

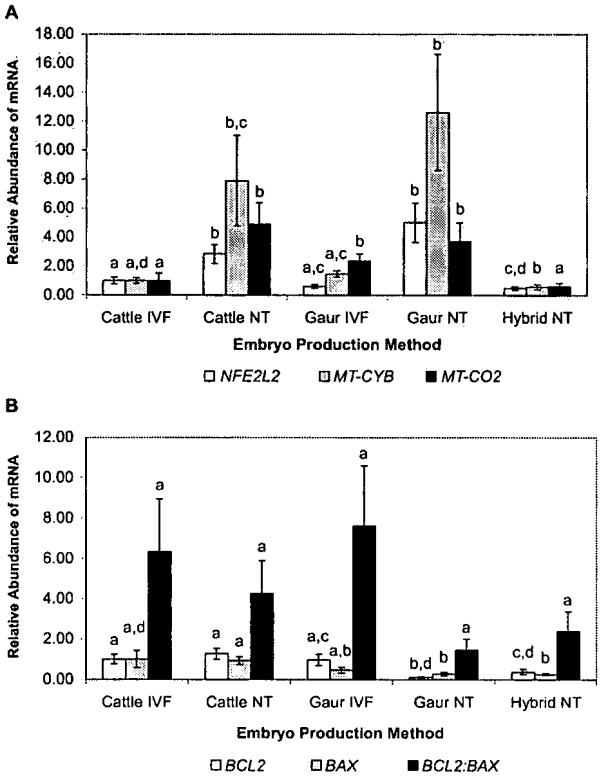

Figure 6. Mitochondrial DNA copy number in gaur NT embryos.